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DOMESTIC AND INTERNATIONAL

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To
The International Preliminary Examining Authority
European Patent Office
D-80298, Munich

Via Fax & DHL Courier
00 49 89 2399 4465
August 9, 2004

Dear Sir,

Re : **RESPONSE TO THE WRITTEN OPINION UNDER RULE 66.3**
International Application : PCT/IN 03/00204
International Filing Date : 30 May 2003 (30-05-2003)
Applicant : Secretary, Department of Atomic Energy et al
Agents' File Ref. : FPAA/288PCT

Please refer to the Written Opinion dated July 14, 2004

In response to the objections raised in the said opinion the applicants wish to provide submissions and amend claims as under:

The Written Opinion is based on the originally filed claims and establishes claims 6 (new claim 1) and 7 (new claim 2) as novel and inventive and having industrial application. Accordingly the applicants wish to amend the original claims by deleting claims 1 to 5. The novel and inventive claim 6 is the principal claim 1 of the amended set of claims. The subsequent claims dependent on claim 6 thus become novel and inventive.

Regarding the finding in the Written Opinion that the kit claims 50 and 51 (new claim 44, 45) are not novel over the cited art the applicants wish to clarify that the said claims are based on the novel and inventive claim 6 (new claim 1) and hence novel and inventive. It is further submitted that the kit claims 45 and 46 have been deleted. Kit claims 42 and 43 as have been amended suitably and claim 43 has been divided into two claims and based on the new claim 1 and its appended claims (new claims 37 to 39).

It is further submitted that the cited art US 5866336 do not teach use of two labeled primers with specific distance of 0-25 nucleotides at which the 3'ends of donor and acceptor labeled primers are brought in the opposite strands upon amplification in amplification product, and signal generation due to energy transfer between them on opposite strands without any further manipulation. The inventors have found that there was no formation of non-specific amplification product in less stringent condition of amplification, yield of the amplification product was 5-20 times higher that of bigger size products and PCR failures were not observed when the method of the present invention was used. As a result of this non-formation of non-specific product and higher yield, many other non-FRET based detection techniques can be employed for detection of the amplification product by the method of present invention. It was also observed that primer dimer formation could also be eliminated and amplification reaction could be completed in very short time in comparison to other methods.

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Primer dimers are generally of the size of the forward primer plus the size of reverse primer minus six to eight base pairs to plus thirty to forty base pairs. It has been found that surprisingly 0-25 base pair separation of the 3' ends of the two primers in the amplification product provide for a method that result in no non-specific product formation unlike the known methods. Due to absence of non-specific product formation the PCR technique used would be faster and more efficient. FRET is a well-known spectroscopic technique. The distance to which FRET takes place is well established. It has been utilized in different applications and in same application in different ways. In nucleic acid amplification field, the same distance relation has been made use of in a number of ways. There are at least seven patents where it has been used. In US patent 5,866,336 the above distance relation has been utilized in a few different ways, but none of them give very high specificity. In the present invention, the same distance relationship has been utilized in an entirely different approach as one of the methods for signal generation and the approach results in very high specificity of amplification signal even in less stringency of amplification thus reducing the amplification reaction failure rate considerably. Thus the approach of the method allows reduction in false positive and false negative result, which is most desirable. None of the prior art methods have this dual advantage. The US patent 5,866,336 teaches only that for a labeled primer to be extended by polymerase without much inhibition, the label should be on a nucleotide at least two nucleotides away from the 3'end of the primer and nothing more than that. It is well known that once a FRET moiety is incorporated in a nucleic acid molecule through a nucleotide, then incorporation of another fluorophore in the same nucleic acid molecule in close distance of the first moiety is inhibited but the distance and the extent to which such inhibition acts is not known. Though the US patent 5,866,336 teaches that a primer labeled with a FRET moiety two nucleotides away from 3'end can be extended by polymerase without much inhibition, what would happen when a pair of such two-labeled primers are used cannot be predicted. Accordingly, the present invention relates to how close the 3'ends of two separately labeled primers could be brought so that the two FRET moieties could be brought close without much inhibition of the amplification process and also how close the two FRET moieties on two primers can be brought. Hence, primer pairs were labeled on penultimate 3'end base with a free 3' hydroxyl group and on nucleotide two nucleotides away from 3'end and used in nucleic acid amplification reaction. It was observed that both types of labeled primers could be extended by polymerase but in case of terminally labeled primer there was more inhibition. Further when two primers labeled on nucleotides two nucleotides away from their 3' ends were used with a separation of their 3'ends 0 nucleotide pair away in the amplification product there was a slight inhibition of the amplification. Hence FRET moiety labeled primer pair can be used in amplification reaction bringing the FRET moieties together separated by 0-25 nucleotide pairs, preferably 2- 20, more preferably 3-20 and most preferably 4-20 nucleotide pairs taking into account the inhibition and reduction in FRET signal with distance. As FRET signal decreases faster with increase in distance a good signal can be generated when the FRET moieties are close enough. It is known that FRET is most intense when the donor and the acceptor moieties are separated by 5 nucleotides, more intense when separated by 10 nucleotides. In view of the above, though a primer pair separately and suitably labeled with FRET moiety such that the FRET moieties come close within their FRET distance can be used, primers

labeled with FRET moiety 2-10 nucleotides away from their 3'ends would be most useful since on amplification the donor and the acceptor moieties on the primers will remain separated by 4 –12 nucleotide pairs in the amplification product thus resulting in good signal. The distance of 12 nucleotide pairs has been chosen on the ground that the two moieties are on two opposite strands, the tenth base on opposite strand comes close to the first base on first strand and the double helical structure of nucleic acid is slightly twisted. Hence FRET moieties on two opposite strands separated by 12 nucleotide pairs will give good signal.

In view of the above the applicants request that the amended claims (1 to 56, pages 82 to 101) and the submission as above be taken into account. The applicant further requests that a second Written Opinion be issued should the above be not acceptable.

Sincerely yours

S. MAJUMDAR
Applicant's Agent

Encl. Amended claims pages 82 to 101.

WE CLAIM :

63 1. A simple and improved method of detection and/or quantitation of target nucleic acid sequence comprising (i) providing at least two oligonucleotides as a pair of primers for amplification of said target sequence; (ii) subject the target sequence to amplification such that the 3' ends of said pair of primers are on two opposite strands and separated from one another by 0-25 nucleotide pairs in the final amplification product; and (iii) carrying out denaturation step and at least a selective annealing step in each cycle.

64 2. A method of claim 1 wherein said denaturation carried out is less than 20 seconds preferably less than 10 seconds, said annealing carried out is less than 15 seconds preferably less than 5 seconds and extension of less than 10 seconds preferably 0 seconds in each cycle for high through put PCR or nucleic acid target analysis.

65 3. A method of anyone of claims 1 to 2 wherein one of the two oligonucleotides of the nucleic acid amplification reaction is labeled at or near preferably near 3'end with a fluorescent or luminescent signaling moiety kept quenched when the labeled oligonucleotide is not incorporated into the amplification product, the same labeled oligonucletide, when incorporated into the amplification product adapted to generate signal through removal of quenching by separating quencher from the signaling moiety.

66 4. A method of anyone of claims 1 to 3 wherein a first oligonucleotide is labeled with a donor MET moiety at or near preferably near 3' end preferably within 2-10 nucleotides away from 3' end and the second oligonucleotide is labeled with an acceptor MET moiety, at or near preferably near 3' end within 2-10 nucleotides away from 3' end, the said donor and acceptor MET moieties belonging to a molecular energy transfer pair and so configured that the donor and the acceptor moieties come within MET distance, 10–80 Angstrom or the nucleotides to which the MET moieties are attached are separated by 2–20 nucleotides preferably by 4 – 12 nucleotides.

67 5. A method of anyone of claims 1 to 4 wherein the labeled oligonucleotides are selected from linear, hair-pin or otherwise configuration.

68 6. A method as claimed in any one of claim 1 to 5 wherein the oligonucleotide labeled with the acceptor moiety or both the oligonucleotides labeled separately with donor or acceptor moiety are provided in quenched condition with quencher or quenchers or by providing them with hair-pin stem structure such that the emission energy of the acceptor or both the acceptor and the donor remain quenched when not incorporated into the amplification product.

69 7. A method as claimed in claim 6 wherein said quenching is achieved following anyone of:

a. at least the oligonucleotide labeled with the acceptor provided in a hair-pin quenched configuration, where the acceptor is provided quenched with a quencher or both the donor as well as the acceptor labeled oligonucleotides are provided in hair-pin quenched configuration so that both the donor and the acceptor moieties are provided quenched with two separate quenchers, the quenchers are provided on the same oligonucleotides and attached at or near the respective 5' ends, the quencher and the acceptor or the donor are on two opposite strands of the stem structure and part of the same oligonucleotide. In the event of formation of hair–pin stem structure the nucleotide to which the donor or the acceptor moiety is attached is complementary and opposite to the nucleotide to which the quencher is attached or the nucleotide to which the donor or the acceptor moiety is attached and the complement of the nucleotide to which the quencher is attached are within five nucleotides, the donor labeled and or acceptor labeled hair-pin quenched oligonucleotides remain quenched when not incorporated into the amplification product;

b. using additional one or two oligonucleotides as the case may be each being labeled separately at or near 5' end with suitable quencher for the acceptor or the donor MET moiety such that one member of the quencher labeled additional oligonucleotide is fully or partly complementary to the acceptor labeled oligonucleotide resulting in quenching of the acceptor when the acceptor labeled oligonucleotide is not incorporated into the amplification product and the second member of the quencher labeled additional oligonucleotide is fully or partly complementary to the donor labeled oligonucleotide resulting in quenching of the donor when the donor labeled oligonucleotide is not incorporated into the amplification product ; and

c. by providing the acceptor labeled oligonucleotide linked to another suitable oligonucleotide complementary partly or fully to this acceptor labeled oligonucleotide and labeled with a quencher at or near its 5' end through a non-nucleotide organic linker or linker and spacer or by providing both the acceptor and donor labeled oligonucleotides linked to two separate additional suitable oligonucleotides fully or partly complementary to the acceptor and donor labeled oligonucleotides respectively through non-nucleotide organic linkers or linkers and spacers and labeled at or near their 5' ends with two quenchers respectively so that the quenchers can quench the acceptor and the donor when the acceptor and the donor labeled oligonucleotides are not incorporated into the amplification product.

To 8. A method of anyone of claims 1 to 7 wherein a first oligonucleotide primer pair selected to amplify a first segment of the target nucleic acid is used at appropriate concentrations, a second oligonuceotide primers selected to amplify a second segment of the first segment at appropriate concentration used in nested PCR when the second oligonucleotide primer pair is any of the labeled oligonucleotide primer pairs of claims 1 to 7.

71 9. A method of anyone of claim 1 to 8 wherein a first oligonucleotide primer pair selected to amplify a first segment of the target nucleic acid is used at appropriate concentration, where one of the said oligonucleotide primer pair is a first member of the primer pairs of any of the claims 1-12 which can be either labeled or unlabeled, a third oligonucleotide unlabeled or suitably labeled for MET and designed to amplify a second segment of the first segment in association with the above first member is nested with signal being generated on said selective amplification of the target nucleic acid.

72 10. A method as claimed in claim 1 to 9, wherein said nucleic acid amplification reaction comprise any known nucleic acid amplification reactions preferably polymerase chain reaction comprising the steps of adding a polymerase, reaction buffer, deoxy nucleoside triphosphates in addition to the effective amounts of amplification primers to the sample, cycling the sample, between at least a denaturation temperature and an annealing temperature, exciting the reaction mixture with the donor exciting radiation or light, measuring the emission of the acceptor MET moiety, optionally that of the donor.

73 11. A method as claimed in anyone of claims 1 to 9, wherein said step of amplifying the target sequence comprise a nucleic acid amplification reaction carried out using one labeled oligonucleotide as one of the two amplification primers of the target sequence amplification reaction along with the other unlabeled primer and a third labeled oligonucleotide for the amplification product, the said labeled oligonucleotide primer being labeled at or near 3'end with a donor or an acceptor MET moiety of a donor-acceptor MET pair and the said third oligonucleotide being labeled at or near 3' end respectively with an acceptor or donor MET moiety of the above MET pair such that upon successful amplification of the target sequence the labeled primer gets incorporated into one of the two strands of the amplification product and the third labeled oligonucleotide hybridizes to this strand of the amplification product into which the labeled oligonucleotide primer get incorporated thus bringing the donor and the acceptor MET moieties within a MET distance 0-20 nucleotides of one another preferably between 1-10/15 resulting in MET between

the two moieties; the above said amplification reaction comprising the steps of adding polymerase, reaction buffer, deoxy nucleoside triphosphates in addition to the effective amounts of the amplification primers to the samples, cycling the sample between at least a denaturation temperature and an elongation temperature, exciting the reaction mixture with the donor exciting radiation or light, measuring the emission of the acceptor MET moiety and optionally the reduction in donor emission, thus allowing detection of nucleic acid target without creating inhibition to amplification reaction and signal measurement without loss of signal.

12. A method as claimed in any one of claims 1 to 11 wherein

a. at least the oligonucleotide labeled with the acceptor is provided in quenched configuration such that the acceptor remains quenched when the acceptor labeled oligonucleotide is not incorporated into or not hybridized to the amplification product, thus reducing the background and remains unquenched in open configuration of the oligonucleotide producing signal when incorporated into or hybridized to the amplification product,

b. the amplified sample is illuminated with light absorbed by the donor MET moiety, and

c. monitoring the sensitized emission from the acceptor and optionally emission from donor of the MET pair moieties.

13. A method as claimed in anyone of claims 1 to 12 wherein a first oligonucleotide of linear or hair-pin configuration labeled with a donor moiety at or near preferably near its 3' end and a second oligonucleotide singly labeled at or near its 3' end with an acceptor moiety capable of absorbing the energy or light emitted by the donor, where the acceptor is selected from a fluorophore or a quencher preferably a quencher including DABCYL or its analogue or nanogold particle black hole quencher, the donor moiety of the first oligonucleotide kept quenched when the first

oligonucleotide is not incorporated into the amplification product either by providing a third oligonucleotide fully or partly complementary to the first oligonucleotide seperately or linked to first oligonucleotide through an organic non-nucleotide linker and labeled at or near its 5' end with a quencher moiety or by providing the first donor labelled oligonucleotide as hair-pin oligonucleotide with a quencher at or near its 5' ends so configured that the quencher comes in close proximity to the donor moiety in its stem structure, the quencher is selected to be capable of absorbing the energy or light emitted by the donor, and selected to be a fluorophore or a non-radiative quencher preferably a quencher including DABCYL or its analogue, nanogold particle, black hole quencher, and not excluding others, the first and second oligonucleotides are the two primers of nucleic acid amplification reaction and are used such that the emission of the donor is quenched by the quencher/acceptor on the second oligonucleotide only in case of formation of primer dimer but in case of specific amplification product formation the above said quencher/acceptor of second oligonucleotide remains at least 10 bases away from the donor moiety incorporated into the amplification product through the first oligonucleotide and at the same time the quencher of first oligonucleotides remains at least 10 bases away from the donor moiety incorporated into the amplification product through the first oligonucleotides thus allowing the donor moiety to emit its characteristic energy or light and signal generation for the detection or quantitation of a target nucleic acid sequence with increased signal to noise.

76 14. A method as claimed in any of claims 1 to 13 where a first oligonucleotide labeled with a donor-1 moiety at or near preferably near its 3' end and a second oligonucleotide singly labeled at or near its 3' end with an acceptor moiety capable of absorbing the energy or light emitted by the donor-1 and a third oligonucleotide singly labeled with a donor-2 moiety at or near its 5' end are provided, the donor-1 is capable of absorbing the energy or light emitted by the donor-2, the third oligonucleotide is fully or partly complementary as long as the complementarity is maintained such that the donor-2 moiety remains quenched by the donor-1 moiety when the first oligonucleotide is not incorporated into the amplification product ; on incorporation of the first oligonucelotide and the second oligonucleotide into the

amplification product the donor-2 moiety labeled third oligonucleotide gets separated from the donor-1 labeled first oligonucleotide and the emission of the donor-2 moiety is measured.

15. A method as claimed in any one of claims 1 to 13 wherein said oligonucleotides are of the length 10 to 40 bases preferably 15 -30 bases and the said hair–pin oligonucleotides comprise anyone of the following:

a. a first oligonucleotide 10 – 40 bases long preferably 15 – 30 bases long fully complementary to the target nucleic acid sequence at the 5' end of which is attached a 5 – 9 bases long second oligonucleotide which may or may not be partially or fully complementary to the target sequence but fully complementary to the 3' end of the first oligonucleotide thus forming a stem and loop structure.

b. a first oligonucleotide of length between 15 – 40 preferably 15 – 30 bases fully complementary to the target nucleic acid sequence at the 5' end of which is attached a second oligonucleotide of length 2 to 12 bases and again at the 5' end of the second oligonucleotide is attached a third oligonucleotide of length 5 – 9 bases, the second and the third oligonucleotide may or may not be partly or fully complementary to the target nucleic acid sequence but the third oligonucleotide being fully complementary to 5 – 9 bases at or near the 3' end of the first oligonucleotide thus forming stem and loop structure.

c. a first oligonucleotide of length between 15 – 40 bases preferably between 15 – 30 bases fully complementary to the target nucleic acid sequence at the 5' end of the said first oligonucleotide is attached a second oligonucleotide of length 5 – 9 bases and at the 3' end of the said first oligonucleotide is attached a third oligonucleotide of length 5 – 9 bases, the second and the third oligonucleotides being fully complementary to each other, may or may not be fully or partly

complementary to the target nucleic acid sequence thus forming a stem and a loop structure of said hair – pin oligonucleotide

d. a first oligonucleotide of length between 15 – 50 preferably 15 – 30 bases fully complementary to the target nucleic acid sequence at the 5' end of a first which is attached a second oligonucleotide of length 10 to 30 bases through a non-nucleotide organic linker the second oligonucleotide may or may not be partly or fully complementary to the target nucleic acid sequence but the second oligonucleotide being complementary to the bases at or near the 3' end of the first oligonucleotide.

16. A method as claimed in anyone of claims 1 to 15 wherein in case of quenched hair-pin, linear or otherwise oligonucleotide the nucleotide to which the donor or acceptor MET moiety is attached is either opposite or within 5 nucleotides away from the nucleotide to which the quencher for the respective donor or acceptor is attached, the donor/ acceptor MET moiety and the quencher being on to opposite stands of the stem.

17. A method as claimed in any one of claims 1 to 16 wherein said oligonucleotides are selected from DNA or RNA or chimeric mixtures or derivatives or modified versions thereof adapted for priming the amplification reaction or hybridizing to the amplified product and are deoxy oligonucleotides, oligonucleotide or peptide nucleic acid or modified oligonucleotides, the target nucleic acid sequence being selected from genomic DNA, mRNA, RNA, cDNA, chemically synthesized DNA or RNA,

18. A method as claimed in anyone of claims 1 to 17 wherein said oligonucleotides are amplification primers (forward and reverse) of polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), allele specific PCR, methylation status PCR, *in situ* PCR, Triamplification, Nucleic acid sequence based amplification, immuno PCR and not excluding others.

19. A method as claimed in anyone of claims 1 to 18 used in real time RNA expression profiling by simultaneously quantitating large number of mRNAs or cDNAs using preferably PCR, RT – PCR, NASBA by using suitably labeled oligonucleotude primer pairs selected from individual mRNAs or cDNAs.

20. A method as claimed in anyone of claims 1 to 19 used in high throughput nucleic acid amplification reactions including PCR, RT-PCR and NASBA comprising providing first oligonucleotide amplification primer for each mRNAs or cDNAs in large number from a pool selected from the sequences near the 5' ends of individual mRNAs or cDNAs and providing as second amplification primer a single common oligonucleotide primer (common for all mRNAs or cDNAs in the pool or sample) complementary to a sequence joined or ligated to 5' end of all mRNAs or cDNAs synthesized by reverse transcription in the pool or sample prior to the subjection to the amplification reaction the oligonucleotide primers being said labeled oligonucleotides primer pairs either first oligonucleotide amplification primer is dual labeled quenched primer of the invention and the second common amplification primer is an unlabeled oligonucleotide or the first oligonucleotide amplification primer is labeled at or near preferably near 3' end with a donor or an acceptor MET moiety and the second common oligonucleotide amplification primer is labeled at or near preferably near 3' end with an acceptor or donor MET moiety respectively which can also be provided quenched.

21. A method as claimed in any one of claims 1 to 19 used in high throughput nucleic acid amplification reactions including PCR, RT-PCR and NASBA comprising providing first oligonucleotide amplification primer for each mRNAs or cDNAs in large number from a pool selected from the sequences near the 3' or 5' ends of a restriction site of cDNAs of individual mRNAs and providing as second amplification primer a single common oligonucleotide primer (common for all mRNAs or cDNAs in the pool or sample) complementary to a sequence joined or ligated to 3' and 5' ends of the restriction fragments of all cDNAs synthesized by reverse transcription in the pool or sample prior to the subjection to the amplification reaction the oligonucleotide primers being said labeled

oligonucleotides primer pairs, either first specific oligonucleotide amplification primer is dual labeled quenched primer of the invention and the second common amplification primer is an unlabeled oligonucleotide or the first oligonucleotide amplification primer is labeled at or near preferably near 3' end with a donor or an acceptor MET moiety and the second common oligonucleotide amplification primer is labeled at or near preferably near 3' end with an acceptor or donor MET moiety respectively, which can also be provided quenched.

22. A method as claimed in anyone of claims 1 to 20 used in RNA splice variant detection, wherein the target nucleotide sequence is a mRNA or a cDNA and the labeled oligonucleotides are either amplification primers (forward and reverse) of many nucleic acid amplification reactions including polymerase chain reaction (PCR), Reverse transcription polymerase chain reaction (RT-PCR), NASBA, one from 3' end of one exon and the other from 5' end of the adjacent exon or one of the two amplification primers of many nucleic acid amplification reactions including PCR, RT-PCR from 3' end of one exon and a probe complemantary to 5'end of the adjacent exon.

23. A method as claimed in anyone of claims 1 to 21 wherein the detectable signal emitted by the acceptor MET moiety/ MET moiety is sizeable and more intense than the signal emitted by the same if there is no MET and the oligonucleotides are so designed that MET moieties come in right proximity such that MET between donor and acceptor moieties can occur and FRET is a preferred form of MET.

24. A method as claimed in anyone of claims 1 to 22 wherein the target nucleic acid sequence is an amplification product or the sequence of infectious disease agent, or genomic sequence of a human, animal, plant or any other organism mutation of which is implicated to the presence of a disorder or disease, or a human, animal or plant genomic sequence, the presence or absence of which is implicated to a disorder or disease, or a human, animal or plant genomic sequence, the presence or absence of which is implicated to susceptibility to an infectious agent, or a plant or any living organism genomic sequence the presence or absence of which is

implicated to a genetic trait or genotyping of the plants, or the living organism, or a genomic sequence of an infectious agent, the presence or absence of which is implicated to strain typing.

25. A method as claimed in anyone of claims 1 to 23 wherein the donor and acceptor pair moieties are selected from any of the donor - acceptor MET \ FRET pairs and the donor moiety is preferably selected from the group consisting of fluorescein, carboxyfluoroscein (FAM), coumarin, 5-(2' amino ethyl) amino napthlein – 1-sulphonic acid (EDANS), rhodamine, anthranilamide, Reactive Red- 4,europium and terbium chelate derivatives, a combination of an organic moiety having a large extinction coefficient of absorption and a fluorophore preferably of high quantum yield. and the said acceptor moiety is preferably selected from the group consisting of fluorescein, fluorescein derivatives like JOE and others, ethidium, texas red, eosin nitrotyrosine, malachite green, pyrene butyrate, Cy- 3 dye, Cy- 5 dye , DABCYL, DABCYL derivatives, rhodamine, rhodamine derivatives, nanogold , black hole quencher and the quencher is selected from the group consisting of DABCYL and its derivatives, rhodamine, nanogold particles, black hole quencher and many other acceptor moieties.

26. A method as claimed in anyone of claims 1, 2, 19 to 21 and 23 wherein the detection and/ or quantitation of amplified target nucleic acid is accomplished by providing double stranded DNA binding fluorescent dye selected from the group preferably consisting of ethidium bromide, CYBER TM GREEN I, pico green, acridine orange, thiazole orange Yo PRO-1 and chromomycin A3.

27. A method as claimed in anyone of claims 1, 2, 19 to 21 and 23 wherein the detection and /or quantitation of amplified target nucleic acid sequences is accomplished by providing the first oligonucleotide primer being labeled with a binding moiety preferably selected from biotin, magnetic particle and microsphere and a hapten or attached to an anchor oligonucleotide directly or through a linker which can be respectively captured by streptavidin or magnet or centrifugation or anti-hapten antibody, capture oligonucleotide or the like and the second oligonucleotide primer

being labeled with a signaling moiety like a fluorophore, rare earth metal chelate, biotin or a hapten, the hapten being detected utilizing antihapten antibody-enzyme conjugate, steptavidin- enzyme conjugate and enzyme substrate, and other conjugates or by using unlabeled second oligonucleotide primer and providing fluorescently labeled nucleotide in appropriate concentration in the reaction mixture.

28. A method as claimed in anyone of claims 1 to 26 wherein the higher signal to noise ratio improvement is achieved by applying hair-pin quenched labeled oligonucleotides of the invention and selecting an amplification product of the size, the size of the forward amplification primer plus the size of the reverse amplifcation primer plus 0-25 bases in the detection of a nucleic acid target sequence using MET/FRET between donor & acceptor moieties.

29. A method as claimed in any one of claims 1 to 28 comprising multiplexing involving multiple pairs of MET labeled oligonucleotides for detection or quantification of multiple targets.

30. A method as claimed in anyone of claims 1 to 28 comprising heterogenous phase detection wherein one of the two amplification primers of many amplificaion reactions including PCR, RT-PCR, NASBA is fixed covalently through 5' end or an internal nucleotide to a solid support through a linker and spacer, and the other amplification primer is in aquaeous phase in contact with the solid phase, the said solid support to which the labeled oligonucleotide is attached is non-porous and transparent or translucent and glass or plastics like polystyrene, polyethylene, polypropylene or dextran and the like and preferably glass or glass wafer.

31. A method of claim 1 to 28 for high throughput hetrogeneous phase target nucleic acid detection wherein first of the two amplification primers of many target nucleic acids for many amplification reactions including PCR, RT-PCR, NASBA are fixed covalently through 5' end or an internal nucleotide to a solid support through a linker and spacer, and the second amplification primers are in aquaeous phase in contact with the solid phase, the said solid support to which the labeled

oligonucleotide is attached is non – porous and transparent or translucent and glass or plastics like polystyrene, polyethylene, polypropylene or dextran and the like and preferably glass or glass wafer.

32. A method as claim 1 to 28 used in high throughput RNA expression profiling by many nucleic acid amplification reactions including PCR, RT-PCR and NASBA not excluding others by providing first oligonucleotide amplification primer for each mRNAs or cDNAs in large number from a pool selected from the sequences near the 5' ends of individual mRNAs or cDNAs, the said first oligonucleotide primers fixed covalently through 5' end or an internal nucleotide to a solid support through a linker and spacer, and the second amplification primers are in aqueous phase in contact with the solid phase and providing as second amplification primer a single common oligonucleotide primer (common for all mRNAs or cDNAs in the pool or sample) complementary to a sequence joined or ligated to 5' end of all mRNAs or cDNAs in the pool or sample prior to the subjection to the amplification reaction the oligonucleotide primers being labeled oligonucleotides primer pairs of the invention, either first oligonucleotide amplification primer is dual labeled quenched primer of the invention and the second common amplification primer is an unlabeled oligonucleotide or the first oligonucleotide amplification primer is labeled at or near preferably near 3' end with a donor or acceptor MET moiety and the second common oligonucleotide amplification primer is labeled at or near preferably near 3' end with an acceptor or donor MET moiety respectively and also can be provided quenched.

33. A method as claim 1 to 28 used in high throughput RNA expression profiling by many nucleic acid amplification reactions including PCR, RT-PCR and NASBA not excluding others by providing first oligonucleotide amplification primer for each mRNAs or cDNAs in large number from a pool selected from the sequences near the 3' or 5' ends of a restriction site of individual mRNAs or cDNAs, the said first oligonucleotide primers fixed covalently through 5' end or an internal nucleotide to a solid support through a linker and spacer, and the second amplification primers are in aquaeous phase in contact with the solid phase and providing as second

amplification primer a single common oligonucleotide primer (common for all mRNAs or cDNAs in the pool or sample) complementary to a sequence joined or ligated to the 3' and 5' ends of all mRNAs or cDNAs restriction digested fragments in the pool or sample prior to the subjection to the amplification reaction the oligonucleotide primers being labeled oligonucleotides primer pairs of the invention, either first oligonucleotide amplification primer is dual labeled quenched primer of the invention and the second common amplification primer is an unlabeled oligonucleotide or the first oligonucleotide amplification primer is labeled at or near preferably near 3' end with a donor or acceptor MET moiety and the second common oligonucleotide amplification primer is labeled at or near preferably near 3' end with an acceptor or donor MET moiety respectively and also can be provided quenched.

34. A method as claimed in anyone of claims 1, 2, 19 to 21 and 23 wherein one or both the primers are provided labeled with a donor or acceptor moiety and a double stranded DNA intercalating dye suitable to act as an acceptor or donor respectively at suitable concentration whereby on successful amplification donor/acceptor labeled primer/primers get incorporated into the amplification product and the double stranded DNA binding (intercalating) dye get intercalated into the amplification product thus bringing it close to the donor or acceptor moiety as the case may be and resulting in MET/FRET which can be measured, more specifically a fluoresecin labeled primer and double stranded DNA binding dye Ethidium bromide are used, where fluorescein is the donor and eithidium act as is the acceptor for FRET to take place between the two.

35. A method of claims 1 to 32 used in closed tube format for detection or quantitation of one or more nucleic acid target sequences

36. A method as claimed in any one of claims 1 to 33 wherein the oligonucleotides used are selected from:

a. 5'-GGG GTA CTA CAG CGC CCT GA – 3'
b. 5'-GGG GTA CTA CAG CGC CCT GA –3'
FAM
c. 5'-GTC CTG GAA GAT GGC CAT GG –3'
d. 5'-GTC CTG GAA GAT GGC CAT GG –3'
JOE
e. 5'-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG –3'
f. 5'-DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG -3'
JOE
g. 5'-DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG –3'
FAM
h. 5'-GCT CAT GGC GCC TGC CTG G –3'
DABCYL

i. 5'-ATG GCC ATG TCC TGG AAG ATG GCC ATG G –3'
j. 5'– GGG GTA CTA CAG CGC CCT – 3'
FAM
k. 5'- GTC CTG GAA GAT GGC CAT GG –3'
Rhod
l. 5'- GTC CTG GAA GAT GGC CAT GG – 3'
JOE
m. 5' GGC AAT GAA AAG CCA CTT CT – 3' as a forward primer to amplify a 50 base pair segment (base position 23, 565-23,614) of *E. coli* genome
n. 5' TTA ACC GGC GAT TGA GTA CC – 3' as a reverse primer to amplify a 50 base pair segment (base position 23,565-23,614) of *E. coli* genome.

37. A kit for use in method of analogous detection and / or quantitation of target nucleic acid sequence or sequences present in a sample comprising

a. a polymerase or polymerases

b. at least two oligonucleotides as a pair of primers for amplification of said target sequence such that after amplifiction the 3' ends of said pair of primers are on two opposite strands and separated from one another by 0-25 nucleotide pairs in the final amplification product;

c. deoxynucleotides in solution (water or buffer) or lyophilized;

d. a reaction buffer for the nucleic acid amplification reaction.

100 38. The kit of claim 37 wherein the oligonucleotides as pair of primers are suitably labeled separately at or near preferably near their 3'ends with a donor or an acceptor MET/FRET moiety and contains free 3'hydroxy group for polymerase to extend, the said donor and acceptor MET moieties belonging to a molecular energy transfer pair and so configured that the donor and the acceptor moieties come close within MET/FRET distance in the amplification product and the nucleotides to which the donor and acceptor moieties are attached are 0-25 nucleotide pairs away.

101 39. The kit of claim 38 wherein at least the acceptor moiety of the acceptor MET moiety labeled oligonucleotide primer is provided quenched when the same is not incorporated into the amplification product or both the donor and acceptor MET moieties of the respectively labeled oligonucleotide primers are provided quenched when the same are not incorporated into the amplification product, the above quenched oligonucleotide primers being the oligonucleotide primers of any of the claims 7 and 15.

102 40. The kit as claimed in anyone of claims 35 to 36 wherein additionally positive control template and suitable MET/FRET labeled primers are also included as control for amplification reaction.

103 41. A kit as claimed in anyone of claims 36 and 37 wherein the first oligonucleotide is labeled near 3' end with a donor MET/FRET moiety and a double stranded DNA intercalating dye capable of absorbing energy or light emitted by the donor moiety and emitting energy or light is also provided.

104 42. A kit as claimed in claim anyone of claims 36 to 40 wherein the first oligonucleotide is labeled near 3' end with an acceptor MET/FRET moiety and a double stranded DNA intercalating dye capable of emitting energy or light on illumination is

provided such that the acceptor moiety is capable of absorbing the energy or light emitted by the intercalating dye and emitting energy or light.

43. A kit as claimed in anyone of claims 36 to 40 comprising multiple oligonucleotide sets of claim for detection and/or/quantitation of multiple target sequences.

44. A kit for the detection of target nucleic acid sequences or sequences using the oligonucleotides used in the methods of claims 5 to 11 wherein the detection and /or quantitation of amplified target nucleic acid sequences is accomplished by providing the first oligonucleotide primer being labeled at or near 5'end with a binding moiety preferably biotin, or magnetic particle or microsphere or a hapten or the like or attached to an anchor oligonucleotide directly or through a linker which can be respectively captured by streptavidin or magnet or centrifugation or anti-hapten antibody, capture oligonucleotide or the like and the second oligonucleotide primer being labeled with a signaling moiety like a fluorophore, rare earth metal chelate, biotin or a hapten, the hapten being detected utilizing antihapten antibody-enzyme conjugate, steptavidin- enzyme conjugate and enzyme substrate, and other conjugates or by using unlabeled second oligonucleotide primer and providing fluorescently labeled nucleotide in the reaction mixture in appropriate concentration.

45. A kit or kits for the detection of target nucleic acid sequences providing all or more components using the oligonucleotides for detection used in methods of claims 5 to 11 wherein the detection and/ or quantitation of amplified target nucleic acid is accomplished by providing double stranded DNA binding fluorescent dye selected from the group consisting of eithidium bromide, CYBER TM GREEN I, pico green, acridine orange, thiazole orange Yo PRO- 1 and chromomycin A3 but not excluding others.

46. The kit as claimed in any one of claims 36 to 43 wherein the oligonucleotides used are selected from:

a. 5'-GGG GTA CTA CAG CGC CCT GA – 3'

b. 5'-GGG GTA CTA CAG CGC CCT GA –3'
FAM

c. 5'-GTC CTG GAA GAT GGC CAT GG –3'

d. 5'-GTC CTG GAA GAT GGC CAT GG –3'
JOE

e. 5'-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG –3'

f. 5'-DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG -3'
JOE

g. 5'-DABCYL-ATG GCC ATC GTC CTG GAA GAT GGC CAT GG –3'
FAM

h. 5'-GCT CAT GGC GCC TGC CTG G –3'
DABCYL

i. 5'-ATG GCC ATG TCC TGG AAG ATG GCC ATG G –3'

j. 5'– GGG GTA CTA CAG CGC CCT – 3'
FAM

k. 5'- GTC CTG GAA GAT GGC CAT GG –3'
Rhod

l. 5'- GTC CTG GAA GAT GGC CAT GG – 3'
JOE

m. 5' GGC AAT GAA AAG CCA CTT CT – 3' as a forward primer to amplify a 50 base pair segment (base position 23, 565-23,614) of *E. coli* genome

n. 5' TTA ACC GGC GAT TGA GTA CC – 3' as a reverse primer to amplify a 50 base pair segment (base position 23,565-23,614) of *E. coli* genome.

47. A method of manufacture of a kit for use in method of analogous detection and / or quantitation of target nucleic acid sequence or sequences present in a sample comprising

a. providing a polymerase or polymerases

b. providing a first oligonucleotide of sequence complementary to the nucleotide sequence flanking a target nucleotide sequence suitably labeled with a donor MET/FRET moiety at or near 3' end.

c. providing a second oligonucleotide of sequence at 5' end of the first nucleotide sequence complementary to the nucleotide sequence flanking the target nucleotide sequence or the segment of the target nucleotide sequence suitably labeled with an acceptor MET/FRET moiety at or near 3' end.

d. providing deoxy nucleotides in solution (water or buffer) or lyophilized.

e. providing a reaction buffer for the nucleic acid amplification reaction.

wherein the first and the second oligonucleotide sequences comprise the two primers (forward and reverse) of many nucleic acid amplification reactions and adapted to generate a detectable signal if the two oligonucleotides get incorporated into two opposite strands of amplified product and come in right proximity an the first and second oligonucleotides are any of the quenched oligonucleotide primers of the claims 1 to 24.

48. A method for high throughput RNA expression preferably for analysis of absolute quantating of mRNAs carried out in homogenous or heterogeneous phase using the method of detection/quantification of target nucleic acid sequence as claimed in anyone of claims 1 to 33.

49. The method as claimed in anyone of preceding claims for the heterogeneous mutation detection comprising two amplification primer oligonucleotides of the invention one being labeled with a donor MET moity near 3' end and the other being labeled with an acceptor MET moiety near 3' end, carrying out target amplification reaction and thermal denaturation analysis of the amplification product or products thus amplified and the same method where the labeled oligonucleotides are also provided in quenched configuration.

50. The method as claimed in anyone of preceding claims used in high throughout nucleic acid amplification reactions including PCR, RT-PCR and not excluding others.

51. The method as claimed in anyone of preceding claims wherein the donor moiety is a combination of an organic moiety having a large extinction coefficient of absorption and a fluorophore preferably of high quantum yield and extinction coefficient.

52. The method as claimed in anyone of preceding claims wherein the higher signal to noise ratio is achieved by applying hair-pin quenched labeled oligonucleotides of the invention in ligase chain reaction.

53. The method as claimed in anyone of preceding claims wherein the higher signal to noise ratio improvement is achieved by applying hair-pin quenched or otherwise quenched labeled oligonucleotides of the invention in the detection of a nucleic acid target sequence using MET/FRET between donor and acceptor moieties on two oligonucleotides designed against one strand of the target sequence.

54. The method as claimed in anyone of preceding claims wherein one or both the amplification primers are labeled at or near 3' end with acceptor or donor moiety or moieties and one of the four deoxynucleotides is provided labeled with the donor or acceptor moiety respectively in appropriate concentrations and composition, and wherein on incorporation of the acceptor labeled primer or primers and the donor labeled nucleotide into the amplification product there is MET/FRET between the donor and the acceptor.

55. The kit as claimed in anyone of preceding claims wherein the polymerases are a reverse transcriptase, T7 RNA polymerase and a DNA polymerase.

56. A method of detection of target nucleic acid sequence, a kit used for the same and its process of manufacture substantially as herein described and illustrated with reference to examples and figures and many modifications thereof.